SARCOSINE DEGRADATION PATHWAY IS INVOLVED IN THE EPIGENETICS OF PROSTATE CELLS

VLADISLAV STRMISKA[1], PETR MICHALEK[1,2], HANA BUCHTELOVA[1], ZUZANA LACKOVA[1], ROMAN GURAN[1,2], SONA KRIZKOVA[1,2], LUCIE VANICKOVA[2], ONDREJ ZITKA[1,2], MARIE STIBOROVA[3], TOMAS ECKSCHLAGER[4], VOJTECH ADAM[1,2], ZBYNEK HEGER[1,2]

[1]Department of Chemistry and Biochemistry
Mendel University in Brno
Zemedelska 1, 613 00 Brno
[2]Central European Institute of Technology
University of Technology in Brno
Purkynova 123, 612 00 Brno
[3]Department of Biochemistry
Charles University
Albertov 2030, 128 40 Prague 2
[4]Department of Paediatric Haematology and Oncology
Charles University, and University Hospital Motol
V Uvalu 84, 150 06, Prague 5
CZECH REPUBLIC

vladislav.strmiska@mendelu.cz

Abstract: It has been shown that sarcosine suplementation stimulates the proliferation of prostate cells and also their invassiveness. In present study we show that enzymes conected with sarcosine conversion to glycine (sarcosine dehydrogenase, pipecolic acid oxidase) are stimulated due to sarcosine treatment. Further, sarcosine treatment increases *S*-adenosylmethioneine-to-*S*-adenosylhomocysteine ratio, which indicates a release and utilization of free methyl groups from sarcosine degradation pathway. We identified the highest induction of global methylation in non-malignant PNT1A cells, but global methylation profiles were altered also in malignant (22Rv1) and metastatic (LNCaP) cells. The influence on methylation changes was further verified using hypomethylating agent 5-azacytidine (5-aza). Co-treatment of prostate cells with 5-aza and sarcosine resulted in decrease in cells invassiveness when compared to treatment with sarcosine alone. This correlates with sarcosine-related hypermethylation of genes involved in cells growth and cell cycle.

Key Words: sarcosine, methylation, prostate, prostate cancer, human cells

INTRODUCTION

Sarcosine is an imino acid and a potential biomarker of prostate cancer (PCa). Concentration of sarcosine is substantially increased during PCa progression to its metastasis (Sreekumar et al. 2009). In its biochemical pathway, sarcosine is formed from dimethylglycine by dimethylglycine dehydrogenase (DMGHD, EC 1.5.8.4) (Metallo 2012) or from glycine by glycine-*N*-methyltransferase (GNMT, EC 2.1.1.20). On the other hand pipecolic acid oxidase (PIPOX, EC 1.5.3.1) or sarcosine dehydrogenase (SARDH, EC 1.5.8.32) can demethylate sarcosine to form glycine while providing free methyl group to methyl-donor *S*-adenosylmethionine (SAM), which is further demethylated into *S*-adenosylhomocysteine (SAH) (Dodt et al. 2000). This can be associated with a methylation of an acceptor (DNA, RNA, neurotransmitters or lipids). Methylation processes can impact a wide array of biological processes, including gene transcription, which could be connected with initiation and progression of PCa. For instance, methylation of cytosine-phosphate-guanine dinucleotides in promoter can inactivate the genes. Although the connection between epigenetics and cancer are well known, there is lack of data regarding the effect of increased amount of sarcosine on methylation status of prostate cells (Ianni et al. 2013).

MATERIAL AND METHODS

Prostatic cell lines

Three human prostatic cell lines were used for an experiment, representing benign and malignant cells: *i*) the PNT1A human cell line established by immortalization of normal adult prostatic epithelial cells by transfection with a plasmid containing SV40 genome with a defective replication origin, *ii*) 22Rv1 which is a human prostate carcinoma epithelial cell line derived from axenograft that was serially propagated in mice after castration-induced regression and relapse of the parental, androgen-dependent CWR22 xenograft, *iii*) LNCaP human cell line estabished from an androgen-sensitive metastasis located in the left supralavicular lymph node. All cell lines used for experiments were purchased from Health Protection Agency Culture Collections (Salisbury, UK).

Culture conditions and treatment protocols

All cell lines were cultured in RPMI-1640 medium with 10% fetal bovine serum (FBS) supplemented by penicillin (100 U/ml) and streptomycin (0.1 mg/ml). The cells were maintained at 37 °C in humidified incubator with 5% CO_2. The exogenous supplementation with sarcosine (10 μM) and azacytidine (10 μM) was initiated after cells reached ~80 % confluence. The cells were harvested after 2; 6 ;12 and 24 h for HPLC-MS analysis and scratch test, after 24; 48 and 72 h for western blotting, and after 24 h for imunofluorescence. All experiments were designed as five biological replicates (n = 5) measured three times at each time point.

Imunofluorescence of sarcosine metabolsim-related enzymes

For imunofluorescence (IF) were culture cells seded into eight-well chamber slides and after 24 h of adherence were treated by sarcosine (10 μM) and azacytidine (10 μM). As a control were used cells without treating. Cells were fixated after 24 h incubation by 4% formaldehyde, permeabilizated by 0.25% Triton X-100, blocked in 5% bovine serum albumin in PBS and imunostained by primary antibody overnight in 4 °C. Detection was accomplished using fluorescein isothiocyanate (FITC)-conjugated or CruzFluor™ 645 (CFL 645) labeled secondary antibodys. DNA staining by Hoechst were used for counter. IF was evaulated by fluorescent microscope for GNMT, SARDH, DMGDH, and PIPOX as a enzymes involved in sarcosine metabolism.

Extraction and quantitation of SAM and SAH

SAM and SAH were extracted in MeOH and acetic acid (80 : 20; *v*/*v*). Solvent was added to the frozen cells followed by slow thawing on ice. After that, the cells wer snap-freezed in liquid nitrogen and thawed on ice again. After three times freez/thaw cycle were samples centrifuged at 9000 × g. The supernatant was transfered to 1.5 ml glass vial and washed with solvent. The quantitation of SAM and SAH was performed using high-performance liquid chromatography with electrospray ionization quadrupole-quadrupole-time-of-flight mass spectrometer (HPLC-ESI-QqTOF MS). The samples were separated on C18 reverse phase column. As mobil phases, water with 0.1% (*v*/*v*) formic acid and methanol with 0.1% (*v*/*v*) formic acid were used.

Global analysis of DNA methylation

The DNA was extracted by the ExtractNow™ DNA Mini Kit and quantified at λ = 260 nm. The global methylation was performed using a methylation DNA quantification kit, incubated with capture and detection antibodies and read by λ = 450 nm. Quantification of global DNA methylation was compared to positive control that had been previously fully methylated. The methylation level depends on global amount of methylated cytosines (5-mC) in samples relative to global cytidine (5-mC + dC).

Wound-healing assay (Scratch test)

The cells were seeded into 16-well plate to reach confluence ~80%. After seeding a pin was used to a scratch and remove cells from a discrete area of the confluent monolayer to form a cell-free zone. After that, cells were treated with sarcosine (10 μM), DNA hypomethylating agent 5-azacytidine (5-Aza, 10 μM). After 6; 12 and 24 h, the micrographs of cells were taken using EVOS FL Auto Cell Imaging System and compared with micrographs obtained in 0 h, using TScratch software.

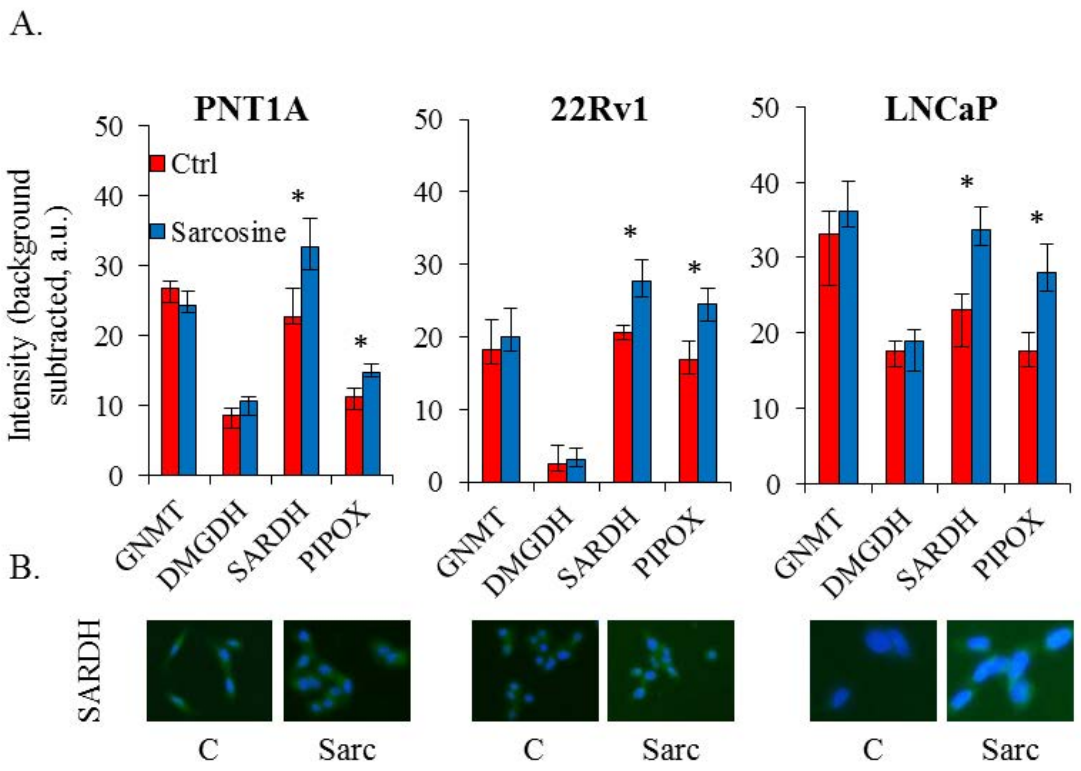
Descriptive statistics

For the statistical evaluation of the results, the mean was taken as the measurement of the main tendency, while standard deviation was taken as the dispersion measurement. Differences between groups were analyzed using paired t-test. Unless noted otherwise, the threshold for significance was $p < 0.05$. For analyses Software Statistica 12 (StatSoft, Tulsa, OK, USA) was employed.

RESULTS AND DISCUSSION

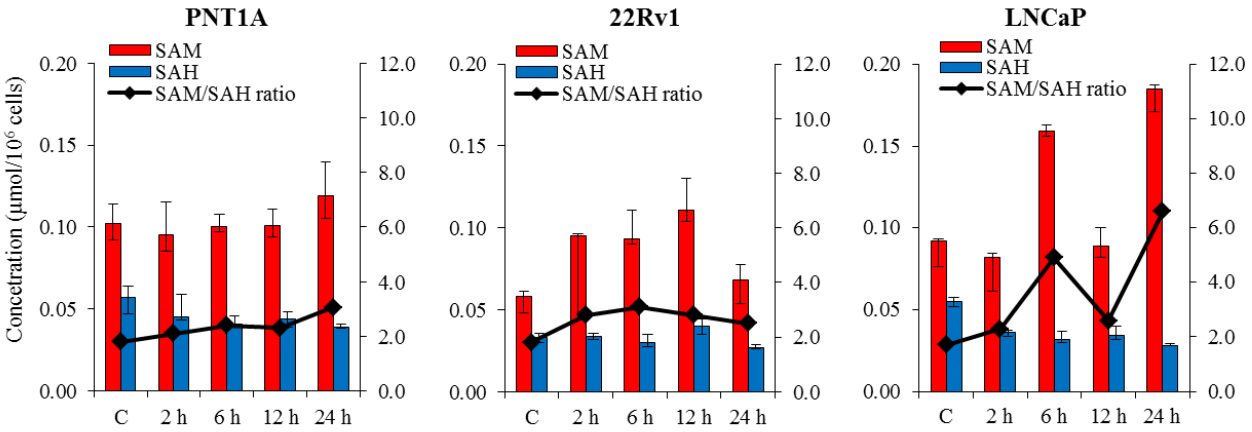
First, we focused on sarcosine stimulatory vs. inhibitory effects on enzymes involved in sarcosine metabolical pathway (GNMT, DMGDH, SARDH and PIPOX). Figure 1 illustrates representative immunofluorescence micrographs of sarcosine metabolism-related enzymes in prostate cells incubated without or with sarcosine (10 μM) together with quantitation of expression of certain enzymes. The stimulatory effects were found for SARDH and PIPOX, which are mainly connected with sarcosine degradation. Hence, it is obvious that an exogenous addition of sarcosine results in its demethylation through sarcosine degradation pathway. Methyl groups can be further used for methylation of various acceptors such as DNA, RNA, etc., which is one of the major hallmarks of cancer development and progression (Suh et al. 2011).

Figure 1 (A) Representative IF micrographs andquantitation of IF of analysed enzymes. (B) IF images of LNCaP cells for SARDH expression after sarcosine treatment compared to control



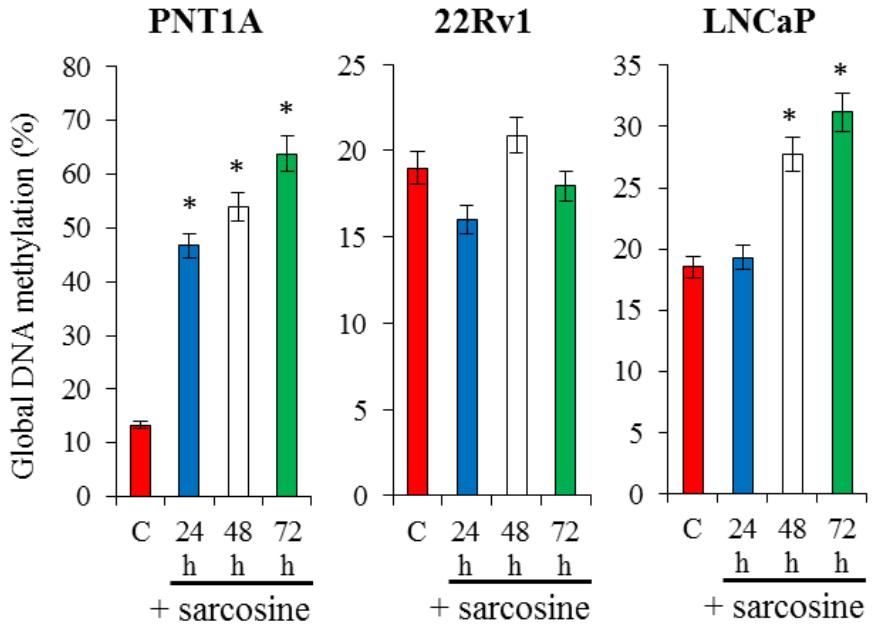
We further focused on estimation of ratio between SAM and SAH. Figure 2 clearly shows that SAM/SAH ratio changed when compared control cells and cells treated with sarcosine. The highest SAM/SAH ratio was determined at LNCaP cells treated with sarcosine after 24 h of incubation. These findings indicate that SARDH/PIPOX degradation of sarcosine had significant stimulatory effects on a formation of methyl-donor SAM, which delivers free methyl groups to the target site (Shukeir et al. 2006). Simultaneously, we identified slight decrease in SAH, which indicate higher needs of prostate cells to maintain SAM activity for transferring the methyl groups.

Figure 2 Values of SAM and SAH and their ratios in PNT1A, 22Rv1 and LNCaP cells non- and supplemented with sarcosine



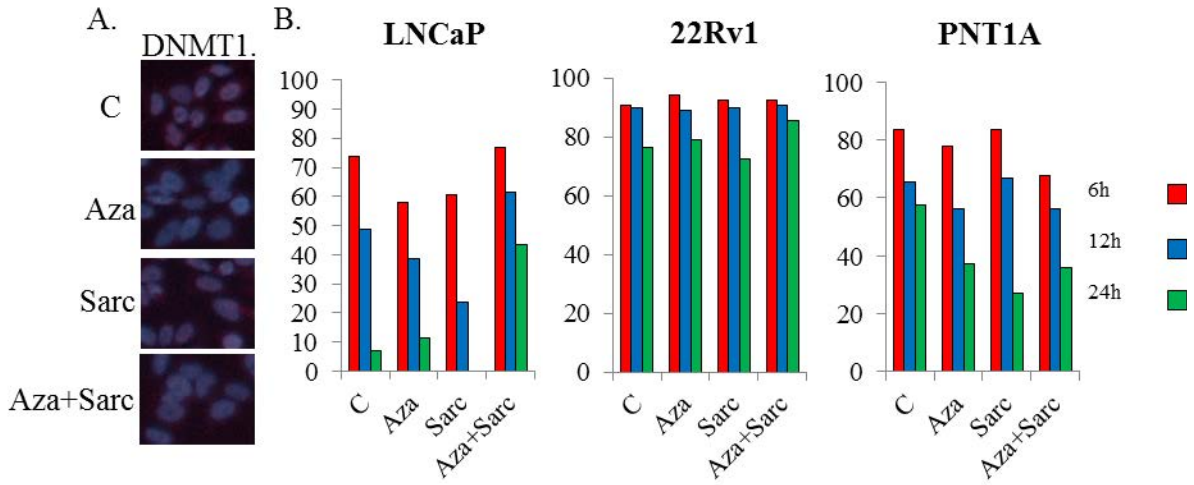
With respect to the obtained data we analyzed global methylation profiles in all tested cells. The highest level of global methylation was found in PNT1A after 72 h treatment (63.8%), which corresponds to the sarcosine-related induction of their proliferation and invassiveness investigated in our previous studies. Methyl groups coming from sarcosine can be used by DNA methyl transferase to methyation of DNA promotors. Overall, we show that sarcosine can efficiently provide free methyl group for DNA methylation processes.

Figure 3 Global methylation index of prostate cell lines treated with sarcosine



To determine whether sarcosine and 5-Aza treatment suppress cell migration was performed (Figure 4). The migration was stopped in the LNCaP by sarcosine treatment after 24 h. The migration was higher after sarcosine supplementation in all three cell lines. That indicate sarcosine as a potential donor of methy group, what can be used for methylation of regulatory genes. In non-malignant cell line PNT1A was not different in migration after 24 h supplementation by 5-Aza and combination of 5-Aza+Sarc. It highlights different metabolism of sarcosine in non-malignant cells and inhibition of methylation. In malignant cells (LNCaP and 22Rv1) after 24 h was proliferation slower in combinant treatment with 5-Aza+Sarc. Hypomethylation caused by 5-Aza probably inhibited methyl group transfer in malignant prostatic cells metaboslim connected with sarcosine metabolism.

Figure 4 (A) Representative IF images for DNMT1 ater treatment. (B) Sarcosine effect on migration of prostate cells



CONCLUSION

The mechanistically increased level of sarcosine stimulates expression of enzymes involved in its metabolism. Sarcosine can hence be utilized as efficient donor of methyl group. Aftervards the methyl groups can be transferred by DNMTs (DNA methl transferases) to promotor areas, which can be hypermethylated. This can result in abnormal transcription with subsequent alterations in cellular proliferation, cell cycle control, etc.

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